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Substrate Specificity of Cyclic Nucleotide Phosphodiesterase from Beef Heart and from *Dictyostelium discoideum*

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The substrate specificity of beef heart phosphodiesterase activity and of the phosphodiesterase activity at the cell surface of the cellular slime mold *Dictyostelium discoideum* has been investigated by measuring the apparent K_m and maximal velocity (V) of 24 derivatives of adenosine 3′,5′-monophosphate (cAMP). Several analogs have increased K_m values, but unaltered V values if compared to cAMP; also the contrary (unaltered K_m and reduced V) has been observed, indicating that binding of the substrate to the enzyme and ring opening are two separate steps in the hydrolysis of cAMP.

cAMP is bound to the beef heart phosphodiesterase by dipole-induced dipole interactions between the adenine moiety and an aromatic amino acid, and possibly by a hydrogen bond between the enzyme and one of the exocyclic oxygen atoms; a cyclic phosphate ring is not required to obtain binding. cAMP is bound to the slime mold enzyme via a hydrogen bond at the 3′-oxygen atom, and probably via a hydrogen bond with one of the exocyclic oxygen atoms. A cyclic phosphate ring is necessary to obtain binding to the enzyme. A specific interaction (polar or hydrophobic) between the base moiety and the enzyme has not been demonstrated. A negative charge on the phosphate moiety is not required for binding of cAMP to either enzyme.

The catalytic reaction in both enzymes is restricted to the phosphorus atom and to the exocyclic oxygen atoms. Substitution of the negatively charged oxygen atom by an uncharged dimethylamino group in axial or equatorial position renders the compound non-hydrolyzable. Substitution of an exocyclic oxygen by a sulphur atom reduces the rate of the catalytic reaction about 100-fold if sulphur is placed in axial position and more than 10000-fold if sulphur is placed in equatorial position. A reaction mechanism for the enzymatic hydrolysis of cAMP is proposed.

cAMP is a key regulator of metabolism, function and growth of many cell types [1]. In mammalian cells cAMP is the second messenger of many hormones; it achieves its function via binding to an intracellular receptor, which is a protein kinase. In the cellular slime mold *Dictyostelium discoideum* cAMP acts as the first messenger [2]; it is excreted by the cells and achieves its function via binding to a cell surface receptor. In *D. discoideum* cAMP induces chemotaxis and cell aggregation (for reviews see [3,4]).

The level of cAMP is controlled by the rate of synthesis catalyzed by adenylate cyclase, the rate of excretion, and the rate of degradation by cyclic nucleotide phosphodiesterase.

In mammalian tissue at least three types of phosphodiesterase have been demonstrated: a calmodulin-dependent enzyme, a cAMP-specific enzyme, and a third form characterized by cGMP activation (for reviews see [5,6]). In the cellular slime molds at least two enzymes have been demonstrated: one enzyme is localized on the cell surface and in the extracellular medium [7,8] and hydrolyzes cAMP and cGMP with about equal rates, and one cGMP-specific enzyme is localized intracellularly [9–11].

cAMP and cGMP analogs have been used systematically to reveal the chemical interactions between cAMP and receptor proteins [12–17], and between cGMP and a cGMP-binding protein and a cGMP-phosphodiesterase [11,18,19]. Recently we have obtained unexpected results with cAMP analogs in *D. discoideum*. Several derivatives were more potent than cAMP, but only at saturating concentrations, and not at threshold concentrations [20]. Furthermore, we observed that several cAMP analogs possessed antagonistic activities against cAMP [21]. Detailed information on the enzymatic degradation of cAMP derivatives is required to understand their action. The interaction between some cAMP derivatives with cyclic nucleotide phosphodiesterase has been described previously [22–25]. In the present work we determined the apparent K_m and the V of 24 cAMP derivatives for two enzymes; the calmodulin-dependent phosphodiesterase from beef heart and the cell surface phosphodiesterase from *D. discoideum*. The results show that the enzymes have widely different specificity. A mechanism for the hydrolysis of cAMP by beef heart phosphodiesterase is proposed, by combining the stereochemical course of the reaction [26], quantum-chemical calculations [27], and the cyclic nucleotide specificity.

Abbreviations. cAMP, adenosine 3′,5′-monophosphate; HPLC, high-performance liquid chromatography.

Enzymes. Cyclic nucleotide phosphodiesterase or 3′,5′-cyclic nucleotide 5′-nucleotidohydrolase (EC 3.1.4.17); 5′-nucleotidase or 5′-ribonucleotide phosphohydrolase (EC 3.1.3.5).

MATERIALS AND METHODS

Materials

[8-^{3}H]cAMP (0.9 TBq/mmol) was obtained from Amersham, snake venom (*Ophiophaga hannah*) was from Sigma,

Dowex AG1X2 was from Serva. The analogs 1, 3, 5, 7, 17, 18, 22–25 (Table 1) were purchased from Boehringer, Mannheim; compound 4 was a generous gift of Dr R. Hanze (Upjohn Cie); compound 6 was kindly supplied by Dr D. Shugar, and compound 20 was a generous gift of Dr F. Eckstein. Compounds 11–14 were kindly supplied by Drs Baraniak and Stec [28]. The analogs 2, 8, 9, 10, 15, 16, 19, 21 were synthesized as described [16, 29–31].

The polarity of the cAMP analogs was determined by high-performance liquid chromatography (HPLC) on a reversed-phase column (Lichrosorp 10 C18, Merck). The mobile phase was 10 mM phosphate buffer, different concentrations methanol, pH 7.5. The polarity of the compounds is expressed as selectivity relative to cAMP, $\alpha = k'(\text{cAMP})/k'(\text{analog})$; k' is the column capacity ratio $k' = (t_r - t_0)\, t_0^{-1}$ where t_r is retention time of a retarded solute and t_0 is the retention time of an unretarded solute. Different concentrations methanol have minor effects on selectivity α, but a pronounced effect on the column capacity ratio k' [32].

Enzyme Preparations

Beef heart phosphodiesterase was purchased from Boehringer, Mannheim. Three different batches have been used with similar results. The cellular slime mold phosphodiesterase was obtained by growing *Dictyostelium discoideum* in association with *Escherichia coli* B/r on a solid medium containing 3.3 g peptone, 3.3 g glucose, 4.5 g KH_2PO_4, 1.5 g $Na_2HPO_4 . H_2O$ and 15 g agar per liter. Cells were harvested in the late log phase in 10 mM KH_2PO_4/NaH_2PO_4, pH 6.0. Bacteria were removed by repeated centrifugations at $100 \times g$ for 4 min. Cells were starved by shaking them in 10 mM phosphate buffer, pH 6.0, at a density of 10^7 cells/ml. After 5 h cells were washed twice in 10 mM phosphate buffer pH 7.5 and suspended in the same buffer at a density of 2×10^7 cells/ml or 4×10^8 cells/ml. Cells were immediately used in the phosphodiesterase assay.

Determination of the K_m *and* V *of the cAMP Derivatives (Mathematics)*

The enzymatic hydrolysis of a substrate S can be written as

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} E + S_p$$

and of a substrate analog A as

$$E + A \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} EA \xrightarrow{k_4} E + A_p$$

where S_p and A_p are the products of respectively S and A.

The rate of hydrolysis of S in the presence of the analog A is given by

$$\frac{-\,d[S]}{dt} = V^s \frac{[S]}{[S] + K_m^s + \left(\frac{K_m^s}{K_m^A}[A]\right)} \qquad (1)$$

where V^s is the V of the enzyme for S.

K_m^s is the K_m for S; $K_m^s = (k_{-1} + k_2)k_1^{-1}$

K_m^A is the K_m for A; $K_m^A = (k_{-3} + k_4)k_3^{-1}$.

We define K_m by its kinetic constants, and not by the substrate concentration at which half-maximal velocity occurs. This definition has the advantage that a non-hydrolyzable analog ($k_4 = 0$) still has a K_m value. We define $[A_{50}]$ as the concentration of substrate analog which reduces the hydrolysis of substrate with 50%. This implies if substituted in Eqn (1)

$$[A_{50}] = \frac{[S] + K_m^s}{K_m^s} K_m^A . \qquad (2)$$

If we measure the hydrolysis of S at a concentration far below its K_m, ($[S] \ll K_m^s$), then $[A_{50}] = K_m^A$.

Therefore we have determined the concentration of a cAMP derivative which reduces the hydrolysis of cAMP at low concentrations by 50%. This concentration equals the K_m of the enzyme for the cAMP derivative. This procedure is only allowed if the enzyme exhibits Michaelis-Menten kinetics. Since the cell surface phosphodiesterase of the slime mold has weak negative cooperativity [33], $[A_{50}]$ equals an apparent K_m.

The second characteristic of the enzyme, V, was determined by measuring the hydrolysis of the analogs at high concentrations. A correction for the various K_m values of the cAMP derivatives was made by applying

$$\frac{-\,d[A]}{dt} = V_A \frac{[A]}{[A] + K_m^A} . \qquad (3)$$

For several derivatives we could not detect any hydrolysis or any inhibition of the hydrolysis of cAMP. Apparently such derivatives do not bind to the enzyme. Therefore, it is unknown how fast this derivative would be hydrolyzed if it could bind to the enzyme; V remains unknown.

Determination of the K_m *and* V *of the cAMP Derivatives (Experimental)*

The K_m of the analogs for the beef heart phosphodiesterase was determined by incubating at 30 °C in a volume of 100 µl containing 50 mM Tris/HCl, pH 7.5, 2 mM $MgCl_2$, 1 µM [^{3}H]cAMP (ca. 2 kBq) 0.25 µg beef heart phosphodiesterase and different concentrations cAMP or cAMP analogs (0.1 µM–1 mM).

The reaction was terminated after 45 min by boiling the samples during 2 min. After cooling, 50 µg snake venom (*Ophiophaga hannah*) was added. Non-hydrolyzed cAMP was removed after 30 min by the addition of 1 ml anion exchanger (1 part AG1X2 and 2 parts H_2O). Samples were shaken for 2 min, centrifuged, and the radioactivity of the supernatant was determined.

The apparent K_m of the analogs for the cell-surface phosphodiesterase was determined by incubating at 20 °C in a total volume of 100 µl containing 10 mM phosphate buffer pH 7.5, 0.1 µM [^{3}H]cAMP (containing ca. 2 kBq), 5×10^5 cells, and different concentrations cAMP or cAMP analogs (0.1 µM–0.25 mM). The reaction was terminated after 0, 2, 5 and 15 min by the addition of 250 µl cold perchloric acid (3.5%, v/v). The lysates were neutralized by adding 115 µl $KHCO_3$ (50% saturated solution at 20 °C), and centrifuged at $8000 \times g$ during 2 min. The supernatant (350 µl) was incubated with 50 µg snake venom during 30 min, and non-hydrolyzed cAMP was removed by the addition of the ion-exchange slurry as described above.

For both enzymes product accumulation was linear with time and enzyme concentration. The interference of the analogs or their degradation products with the 5′-nucleotidase step was investigated by replacing [^{3}H]cAMP with [^{3}H]-

5′AMP in the above-described incubation procedure. Inhibition of the hydrolysis of [^{3}H]5′AMP by the cAMP analogs or their degradation products has not been observed.

The V of the analogs was determined by measuring their degradation with HPLC. The equipment consisted of a Beckman 100A pump and a Laboratory Data Control UVIII monitor at 254 nm. The column used was the anionexchanger Partisil 10-SAX (Whatman) and the mobile phase composition was 50 mM KH_2PO_4, 15% propan-1-ol, 5% methanol, pH 5. This composition excludes most of the hydrophobic interactions between solutes and the stationary phase [34], thus separating almost exclusively on the charge, which is the main difference between cyclic nucleotides and nucleotides. For the beef heart phosphodiesterase the incubation took place at 30 °C in a total volume of 50 µl containing 50 mM Tris/HCl, pH 7.5, 2 mM $MgCl_2$, 2.5 mM cAMP or cAMP analogs and 30 µg phosphodiesterase. At 5 min or longer intervals 1 µl of this mixture was injected in the separation system described above. The degree of degradation was quantified by measuring the decrease of the peak area of the substrate. The half-life of cAMP was 12 min. If required, analysis was continued up till 24 h.

For the slime mold phosphodiesterase the incubation at 20 °C contained in a total volume of 50 µl: 10 mM phosphate buffer, pH 7.5, 0.2 mM cAMP or cAMP analogs and 10^7 cells. The reactions were terminated after 0, 2, 5, 7.5, 10, 20 and 30 min by centrifugation for 5 s at $8000 \times g$ and adding 25 µl of the supernatant to 75 µl cold ethanol. After centrifugation for 2 min at $8000 \times g$ 15 µl of the supernatant was analysed on the separation system described above. The half-life of cAMP varied between 5 and 10 min.

RESULTS AND DISCUSSION

Selection of cAMP Derivatives

The interaction of a small molecule with a protein is mainly electrostatic [35]. The types of interaction include: ion-ion interactions, dipole interactions such as hydrogen bonds, hydrophobic interactions such as π-electron stacking, and changes of free energy due to solvophobic effects.

cAMP can form several hydrogen bonds with its surrounding medium (e.g. water, protein). One hydrogen bond cannot be formed any longer in each of the derivatives 2–4, 7–10 (Fig. 1 a). The derivatives 5 and 6 were used to change the syn-anti equilibrium, which is 1 : 1 in cAMP [36], to the syn conformation [37]. Compound 8 was used to introduce a bulky substituent close to the site of the catalytic reaction.

In derivatives 11 and 12 the electron density distribution of the negative charge is no longer symmetrically located but preferentially on oxygen. Derivatives 13 and 14 were used to remove the negative charge; thus the double bond oxygen is regio-selectively fixed. These derivatives (11–14) may reveal the stereochemical orientation and involvement of the exocyclic oxygen atoms during the hydrolytic reaction.

The derivatives 1, 15–18 (Fig. 1 c) form a sequence of decreasing polarizability (15, 1, 16, 18, 17 [38]) and have decreasing polarizing power (18, 17, 1, 16, 15 [38]). The derivatives 19–25 are not cyclic nucleotides. Some of them (19–21) can mimic the ribose-cyclic phosphate moiety of cAMP and bind to their receptor proteins [14, 39] (Fig. 1 d)

The polarity of all derivatives has been measured by high-performance liquid reversed phase chromatography. These data might reveal hydrophobic interactions between cAMP and phosphodiesterases.

Table 1. *List of cAMP derivatives*

No.	Name
1	adenosine 3′,5′-monophosphate (cAMP)
2	adenosine-N^1-oxide 3′,5′-monophosphate
3	6-chloropurineriboside 3′,5′-monophosphate
4	7-deazaadenosine 3′,5′-monophosphate
5	8-bromoadenosine 3′,5′-monophosphate
6	8-hydroxyisopropyladenosine 3′,5′-monophosphate
7	2′-deoxyadenosine 3′,5′-monophosphate
8	2′-deoxy 2′-*O*-(2,4-dinitrophenoxy)-adenosine 3′,5′-monophosphate
9	3′-deoxy 3′-aminoadenosine 3′,5′-monophosphate
10	5′-deoxy 5′-aminoadenosine 3′,5′-monophosphate
11	adenosine 3′,5′-monophosphorothioate Rp-isomer
12	adenosine 3′,5′-monophosphorothioate Sp-isomer
13	adenosine 3′,5′-monophosphodimethylamidate Rp-isomer
14	adenosine 3′,5′-monophosphodimethylamidate Sp-isomer
15	benzimidazoleriboside 3′,5′-monophosphate
16	purineriboside 3′,5′-monophosphate
17	inosine 3′,5′-monophosphate
18	guanosine 3′,5′-monophosphate
19	5′-deoxy 5′-aminoadenosine 3′-monophosphate
20	adenosine 5′-monophosphate-methylester
21	myoinositol 1,2-monophosphate
22	adenosine 5′-monophosphate
23	adenosine 3′-monophosphate
24	adenosine
25	adenine

Molecular Events Shown by Changes of K_m *and* V

The V of an analog reveals the effect of chemical modification of cAMP on the rate of the catalytic reaction, once the analog is bound to the enzyme. The K_m of the analogs gives information on the binding of the analogs to the enzyme. However, the K_m does not equal the dissociation constant of the enzyme for the analogs, since the proportions of k_{-3} and k_4, as well as the proportioning of k_{-1} and k_2 are unknown. If V is not changed by the modification (k_2 and k_4 are identical) then the change of K_m represents a variation of the binding affinity of the analog. Data on K_m values should be treated with caution if V is strongly influenced by the modification.

In Fig. 2 the inhibition of the hydrolysis of radioactive cAMP by non-radioactive cAMP (compound 1) and three cAMP derivatives (compounds 2, 9 and 10) is shown. It reveals several differences between the slime mold enzyme and the beef heart enzyme. The apparent K_m for cAMP of the slime mold enzyme varied between 0.5–1 µM, whereas the apparent K_m of the beef heart enzyme is approximately 25 µM. Further, these enzymes have considerably different K_m values for the cAMP derivatives relative to cAMP. The hydrolysis of cAMP and these three analogs was measured at high concentrations (Fig. 3). Although the K_m of the beef heart enzyme for compound 2 was about 70-times higher than the K_m for cAMP, their rate of hydrolysis differ only by a factor of 5. By applying Eqn 3 it appears that the V of compound 2 is only three times less than the V of cAMP.

The other derivatives were treated in the same way; the results are summarized in Table 2. The derivatives which inhibited the hydrolysis of [^{3}H]cAMP by the beef heart phosphodiesterase, but which were not hydrolyzed, were investigated for competitive inhibition. The hydrolysis of different concentrations [^{3}H]cAMP was measured at different concentrations of the analogs. All analogs were competitive inhibitors (data not shown).

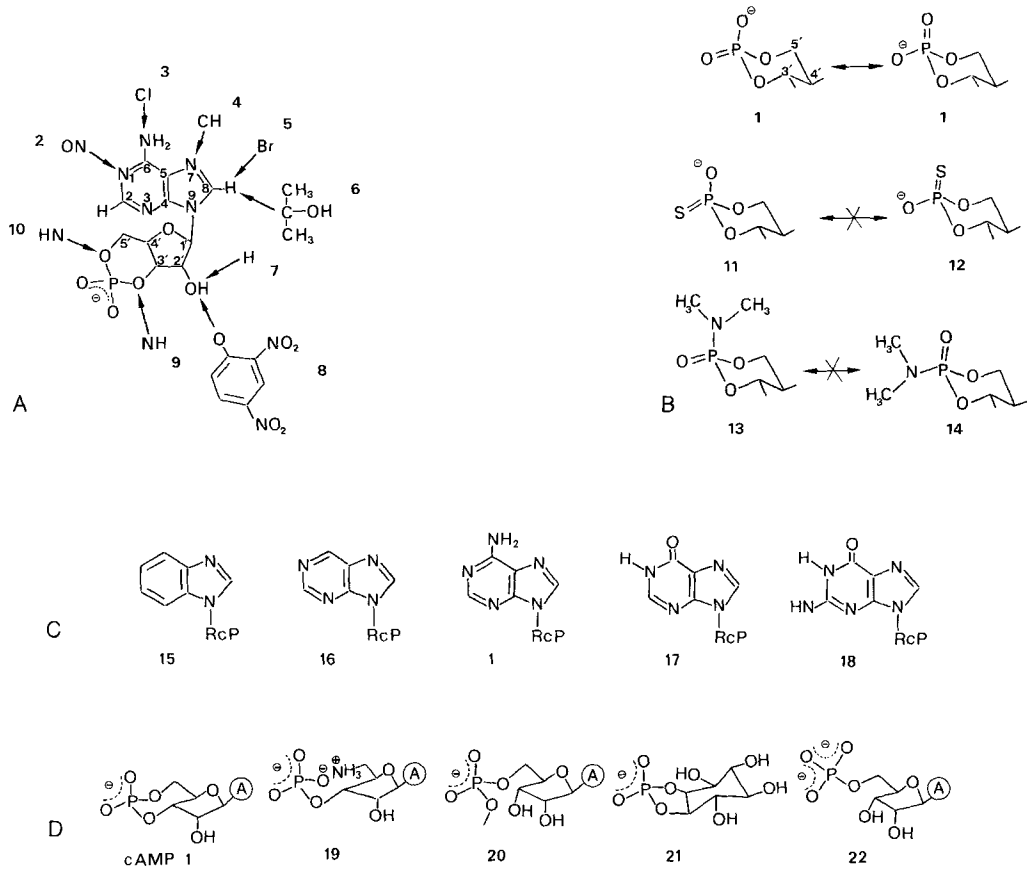


Fig. 1. *Structures of the cAMP analogs*

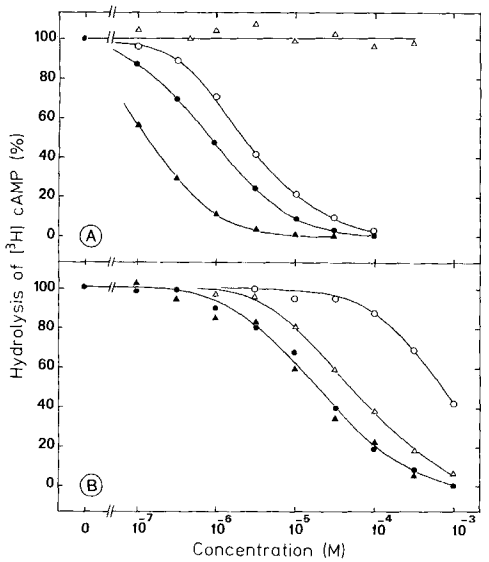


Fig. 2. *Inhibition of the hydrolysis of [^{3}H]cAMP by cAMP derivatives.* (A) Cell-surface phosphodiesterase of *D. discoideum*; (B) beef heart phosphodiesterase. (●) Compound 1; (○) compound 2; (▲) compound 9; (△) compound 10

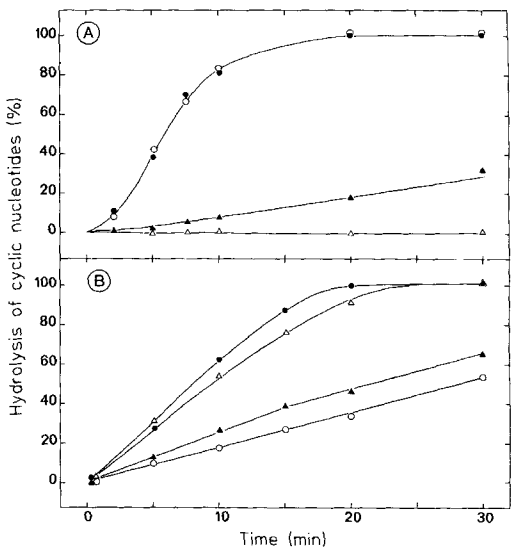


Fig. 3. *Hydrolysis of cAMP and cAMP derivatives by cell-surface phosphodiesterase of* D. discoideum *(A) or by beef heart phosphodiesterase (B).* (●) Compound 1; (○) compound 2; (▲) compound 9; (△) compound 10

Specificity of the Cell-Surface Phosphodiesterase of D. discoideum

Table 2 reveals that most of the cAMP analogs modified in the base or ribose moiety (2–10) have a V comparable to cAMP. Exceptions are compound 6, which has a bulky substituent on the 8-position, compound 9, for which V could not be calculated, and compound 10. This indicates that the base moiety and 2′-hydroxy are not directly involved in the catalytic reaction. The K_m values of these analogs (2–10) suggest

Table 2. *Specificity of phosphodiesterase and cAMP receptors relative to cAMP*

K'_m is the apparent K_m of the analog relative to cAMP: $K'_m = K_m$ (analog)/K_m (cAMP). V' is the maximal velocity of the hydrolysis of the analog relative to cAMP; $V' = V$ (analog)/V (cAMP). The polarity of the analogs is presented as selectivity to cAMP; $\alpha = k'$ (cAMP)/k' (analog) (see Materials and Methods). sm, slime mold; bh, beef heart

No.	Phosphodiesterase				Polarity
	K'_m		V'		α
	sm	bh	sm	bh	
1	1	1	1	1	1
2	2.8	71	1.0	0.5	6.25
3	2.5	7.8	0.7	1.1	0.43
4	0.8	6.0	1.5	0.9	0.86
5	6.5	2.1	2.2	0.2	0.45
6	40	16	0.07	0.01	0.51
7	4.5	2.5	0.4	0.8	1.20
8	115	1.9	0.4	0.12	0.035
9	> 2500	3.3	–[a]	0.9	1.0
10	0.07	0.9	0.1	0.4	1.96
11	> 2500	14	–[a]	$< 10^{-4}$	0.76
12	220	6	$< 10^{-2}$	0.015	0.51
13	450	80	$< 10^{-2}$	$< 10^{-4}$	0.37
14	23	15	$< 10^{-2}$	$< 10^{-4}$	0.19
15	7.6	9.2	1.0	1.1	0.33
16	2.9	3.2	0.5	1.8	1.20
17	12	0.3	0.9	1.3	3.57
18	2.2	0.2	0.7	0.7	3.70
19	> 2500	56	–[a]	$< 10^{-4}$	14.3
20	> 2500	32	–[a]	$< 10^{-4}$	1.72
21	> 2500	30	–[b]	–[b]	–[b]
22	> 2500	125	–[a]	$< 10^{-4}$	33
23	> 2500	57	–[a]	$< 10^{-4}$	10.0
24	> 2500	79	–[a]	$< 10^{-4}$	0.81
25	> 2500	70	–[a]	$< 10^{-4}$	0.88

[a] V could not be calculated due to the absence of binding of the analog to the phosphodiesterase.

[b] Hydrolysis and polarity of this compound has not been measured due to the absence of a chromophor.

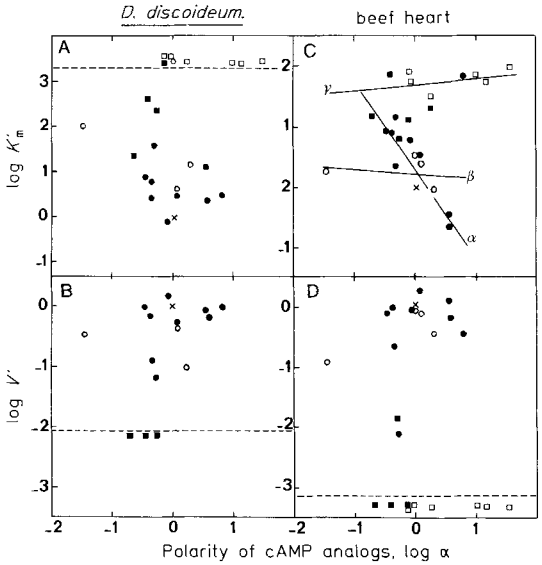


Fig. 4. *Correlation between the polarity of cAMP and cAMP derivatives versus* K_m *and* V. (A, B) Cell-surface phosphodiesterase of *D. discoideum*; (C, D) beef heart phosphodiesterase. (A, C) K_m relative to the K_m of cAMP; (B, D) V relative to the V of cAMP. The lines in C are based on linear regression analysis of analogs modified in the base (α), analogs modified in the ribose (β), and non-cyclic analogs (γ). (×) cAMP; (●) analogs modified in the base (compounds 2–6, 15–18); (○) analogs modified in the ribose (compounds 7–10); (■) analogs with modifications in the exocyclic oxygen atoms (compounds 11–14); (□) non-cyclic analogs (compounds 19–20, 22–25)

that the adenine moiety, 2′-hydroxy and 5′-oxygen also are not directly involved (e.g. via hydrogen binding) in the binding of cAMP to the enzyme. A possible explanation of the low K_m value of compound 10 has been given in the previous section. Compound 8 has a very bulky substituent at the 2′-position, close to the catalytic site, and it has a high K_m but a V comparable to that of cAMP, which indicates that hydrolysis is normal, but that binding is impaired. Modification of the 3′-oxygen by an amino group results in the loss of binding to the enzyme, which strongly suggests that cAMP is bound to the enzyme via an essential hydrogen bound at the 3′-position. Although hydrogen bonds between adenine and enzyme seem to be unlikely, several other interactions may take place. Stacking interactions between the π-electrons of adenine and an aromatic amino acid is unlikely, since the order of K_m or V of compounds 1, 15–18 does not follow the order of decreasing polarizability (15, 1, 16, 18, 17 [38]) or decreasing polarizing power (18, 17, 1, 16, 15 [38]). Since a correlation between K_m or V and the polarity of the analogs is clearly absent (Fig. 4A and B), changes of free energy due to solvophobic effects are also unlikely.

In contrast to the relatively low specificity in the adenine moiety, modification of the exocyclic oxygen atoms (compounds 11–14) has a strong effect on the interaction with the enzyme. Hydrolysis of these compounds has not been observed, indicating that both exocyclic oxygen atoms are involved in the catalytic reaction. The potency of the non-charged compound 14 to inhibit the hydrolysis of cAMP strongly suggests that a charge-charge interaction between cAMP and the enzyme is not required for the binding of cAMP. Furthermore these compounds reveal a high stereospecific recognition of the exocyclic oxygen atoms by the enzyme. None of the non-cyclic nucleotides (19–25) bind to the enzyme, and consequently none of them was hydrolyzed.

Summarizing, we propose that cAMP is bound to the phosphodiesterase of *Dictyostelium discoideum* via hydrogen bonds at the 3′-oxygen position, and one of the exocyclic oxygens (probably the double bond oxygen in axial position), but not by a charge-charge interaction. A distinct hydrophobic interaction between the base moiety and the enzyme seems unlikely. Hydrolysis requires a correct steric orientation of the exocyclic oxygen atoms.

Specificity of the Beef Heart Phosphodiesterase

cAMP analogs modified in the base or ribose moiety (compounds 2–10) have a V comparable to cAMP, except the compounds with bulky substituents (5, 6 and 8).

Most of these analogs (2–10) have a K_m comparable to cAMP. The high K_m of compound 2 may indicate a hydrogen bond at N^1 with the enzyme; however, this is not shown by cIMP (compound 17), cGMP (compound 18), and compound 15. Although cAMP is hydrolyzed at the 3′-oxygen atom, the results with compound 9 (3′-oxygen replaced by an amino group) show that a direct chemical interaction between the enzyme and the 3′-oxygen atom during binding and hydro-

lysis is absent. This observation is in strong contrast to the slime mold enzyme.

Modifications of the exocyclic oxygen atoms (compounds 11–14) have a strong effect on the rate of hydrolysis; compound 12 is hydrolyzed 70-times slower than cAMP, hydrolysis of the other compounds has not been observed (hydrolysis is at least 10000-fold slower than the hydrolysis of cAMP). In contrast to the drastic effects on the hydrolyzability, these compounds (11–14) bind to the enzyme with apparently high affinity. However, these results should be interpreted very cautiously, since the relative proportioning of k_{-1} to k_2 is unknown. Also the non-cyclic nucleotides (compounds 19–25) bind to the enzyme but hydrolysis has not been observed.

Hydrogen bonds between the adenine moiety and the beef heart enzyme are unlikely. The results of the K_m values of compounds 1, 15–18 may suggest stacking interactions of the π-electrons of adenine via a polarization of the π-electrons of an aromatic amino acid, since the order of decreasing affinity (increasing K_m values) corresponds with the order of decreasing polarizing power ($18 > 17 > 1 > 16 > 15$; [38]). Fig. 4C reveals that analogs which are modified in the base moiety have an increased affinity for the enzyme if they are more polar. This correlation between polarity of the analog and K_m value is absent in analogs not modified in the base moiety, which confirms the assumption that the adenine moiety is bound to the enzyme by dipole-induced dipole forces via stacking interactions due to polarization of an aromatic amino acid. Hydrophobic interactions do not seem to be involved in the catalytic reaction of this enzyme (Fig. 4D).

The phosphodiesterase activities of slime molds and beef heart differ significantly in the following aspects: (a) their apparent K_m values for cAMP, (b) the involvement of the adenine moiety in the binding of cAMP to the enzyme, (c) the involvement of the 3′-oxygen atom in both the binding and the catalytic reaction, and (d) the binding of non-cyclic analogs. This last observation might explain why all the classical phosphodiesterase inhibitors (caffeine, theophylline) fail to inhibit the slime mold phosphodiesterase [40].

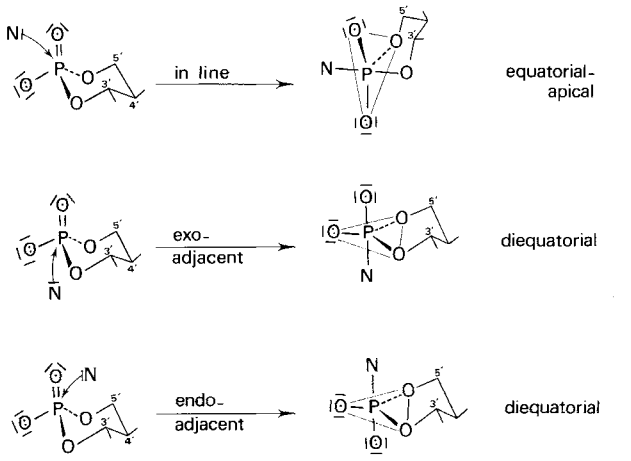
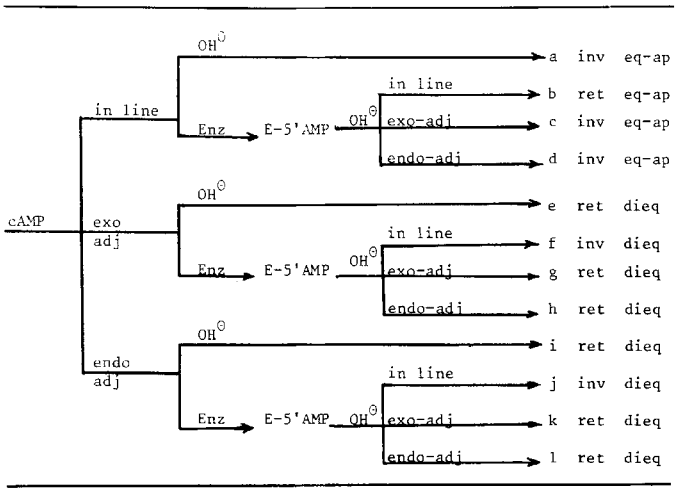


Fig. 5. *Nomenclature of nucleophilic reactions and of conformations of the cyclophosphate ring.* N is nucleophile ($OH^{\ominus}$ or enzyme)

Table 3. *Possible routes for the hydrolysis of cAMP by phosphodiesterase* In line, exo-adjacent and endo-adjacent are the three reaction mechanisms; $OH^{\ominus}$ and Enz (enzyme) are the two nucleophiles; E-5′AMP is the enzyme-5′AMP complex; inv and ret are respectively inversion and retention at phosphorus eq-ap is an equatorial-apical-positioned cyclophosphate ring; dieq is a diequatorially-positioned cyclophosphate ring



Reaction Mechanism of Hydrolysis

Several cAMP analogs have relatively unaltered V values, while the K_m is increased significantly (compound 8 with slime mold and compound 2 with beef heart); the opposite, unaltered K_m and reduced V, has also been observed (compound 14). This demonstrates that hydrolysis of cAMP proceeds in at least two steps: binding of cAMP to the enzyme, and ring opening catalyzed by the enzyme.

Enzymatic hydrolysis of phosphate ester bonds generally takes place via nucleophilic substitution reactions with phosphate intermediates arranged in trigonal bipyramidal configuration [41]. The nucleophilic attack may take place in three directions (Fig. 5): opposite the leaving group (in line), or opposite a ligand which is not the leaving group (endo-adjacent or exo-adjacent, Fig. 5). There are two possibilities for the nature of the nucleophile, i.e. a water molecule, or a site of the enzyme. The product of the reaction is 5′AMP if water is the nucleophile; however, if the enzyme is the nucleophile the product is an enzyme-5′AMP complex. This complex is subsequently cleaved by a nucleophilic attack of a water molecule with three possibilities (in line, endo-adjacent or exo-adjacent). Therefore, twelve different routes are possible for the hydrolysis of cAMP by phosphodiesterase (Table 3).

Burger et al. [26] showed that beef heart phosphodiesterase hydrolyzes cAMP with inversion at phosphorus. This eliminates seven hydrolysis routes (Table 3).

Recently, Van Ool and Buck [27] published quantum chemical calculations on cAMP-enzyme intermediates with trigonal bipyramidal configurations. Their calculations show that (a) the trigonal bipyramidal configuration of cAMP with a diequatorial cyclophosphate ring is about 100 kJ/mol lower in energy than the equatorial-apical ring-positioned intermediate of cAMP; (b) the intermediate of compound 12 is about 525 kJ/mol lower in energy than the intermediate of compound 11 if they both have a diequatorially positioned cyclophosphate ring; (c) the energy difference is only 53 kJ/mol in favour of compound 12 if the intermediates have an equatorial-apical-positioned cyclophosphate ring. Our observation that only compound 12 is hydrolyzed strongly suggests

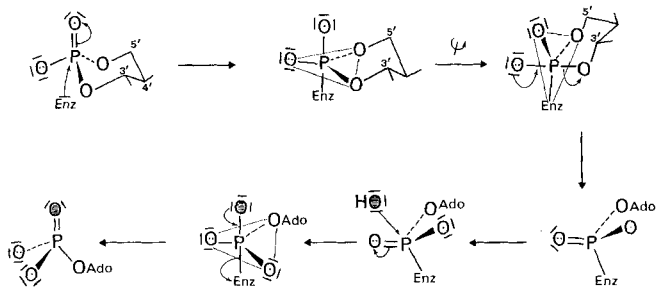


Fig. 6. *Model of the reaction mechanism of the hydrolysis of cAMP by beef heart cyclic nucleotide phosphodiesterase*

that the intermediate has a diequatorially-positioned cyclophosphate ring. This excludes another three hydrolysis routes (Table 3). Note that Van Ool and Buck conclude that cAMP is hydrolyzed via route c or d (Table 3). This result was based on the report that compounds 11 and 12 are hydrolyzed at the same rate [42]; therefore, they had to conclude that the intermediates of 11 and 12 have an equatorial-apical positioned cyclophosphate ring. Our observation that only the (Sp)-diastereoisomer is a substrate has been confirmed by others (personal communication of Dr Eckstein to Dr Jarvest, cited in [43] (p. 464). Note also that Jarvest et al. [43] proposed that cAMP is hydrolyzed via route a (Table 3), which is excluded in the present paper. The quantum-chemical calculations by Van Ool and Buck were not available at that time.

The two remaining routes (f and j) differ in an exo- or endo-attack of the enzymatic site in the first nucleophilic reaction. The only—not very strong—argument in favour of the route f (exo-attack) might be that an endo-attack is constrained spatially. This might be especially important if cAMP is bound to the catalytic site in the syn conformation [25]. Therefore, the most likely reaction mechanism for the hydrolysis of cAMP by beef heart phosphodiesterase consists of the binding of cAMP to the enzyme followed by two nucleophilic substitution reactions (Fig. 6). The first reaction is an exo-attack of a nucleophile of the enzyme (e.g. serine or threonine). This leads to an intermediate with the cyclophosphate ring in the favourable diequatorial conformation. After pseudorotation the 3′-phosphate bond is cleaved leading to an enzyme-5′AMP intermediate. The second reaction is an in line attack of a water molecule by which the enzyme-5′AMP bond is cleaved.

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